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Strain-Specific Differences in the Attachment of *Listeria monocytogenes* to Alfalfa Sprouts

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ABSTRACT

Contamination of fresh produce with *Listeria monocytogenes* has resulted in outbreaks of systemic listeriosis and febrile gastroenteritis. Recalls of alfalfa sprouts have occurred due to contamination with *L. monocytogenes*. Alfalfa sprouts were used as a preharvest model to study the interaction with this human pathogen. Seventeen strains were assessed for their capacity to colonize alfalfa sprouts, and strain-specific differences (not related to source, serotype, or lineage) were revealed when the sprout irrigation water was changed daily. Two of the strains colonized and attached to the sprouts very well, reaching levels of more than 5 log CFU per sprout. The remaining strains varied in their final levels on sprouts between less than 1 to 4.7 log CFU per sprout. All of the *L. monocytogenes* strains grew to equivalent levels on the sprouts when the irrigation water was not changed, suggesting the differences observed with regular changing of the water resulted from differences in attachment. Further analysis of the best colonizing strains indicated that only between 0.3 and 1 log CFU per sprout could be removed by additional washing of the sprout, and the presence of normal sprout bacteria did not compete with the *L. monocytogenes* strains on the sprouts. The poorest colonizing strain was able to grow in the irrigation water during the experiment but could not attach to the sprouts. Microscopic examination of the sprouts with *L. monocytogenes* expressing the green fluorescent protein indicated that *L. monocytogenes* was associated with the root hairs of the sprouting alfalfa, with few to no cells visible elsewhere on the sprout.

Listeria monocytogenes, a gram-positive, saprophytic bacterium often found in soil and agricultural environments, causes approximately half of the reported deaths that result from foodborne illness *(12)*. Outbreaks of listeriosis and recalls of fresh or processed produce have occurred from *L. monocytogenes* contamination *(3, 4, 6, 9, 18)*. Despite the environmental niches of *L. monocytogenes*, little is known of the physiology of the bacterium when it is in a produce or plant environment. Cases of foodborne illness that result from the consumption of alfalfa sprouts have been associated mostly with *Salmonella enterica* and *Escherichia coli (23)*. Although no reported cases of listeriosis have ever been traced to contaminated alfalfa sprouts, contamination of sprouts with *L. monocytogenes* has resulted in product recalls, and *L. monocytogenes* has been isolated from retail alfalfa sprouts *(1, 2, 5, 23, 28)*. In addition, cases of listeriosis have been traced back to alfalfa tablets *(13)*.

As a result of sprout-associated outbreaks of *S. enterica* and *E. coli*, it is common practice in the sprouting industry to sanitize the seed with 3% (30,000 ppm) $Ca(OCl)_2$. Although this practice decreases the bacterial load on the seeds, empirical evidence suggests it decreases the number of bacterial pathogens on sprouts; however, pathogens have been shown to survive the treatment *(11, 16, 29)*. In addition, the integrity of the seed has a role in the number of bacteria on the seed and the efficacy of sanitization. Wrinkled or damaged seeds have been shown to harbor many more bacteria in general and are more resistant to chemical sanitization with $Ca(OCl)_2$ *(11)*.

Previously, we modeled the attachment of *L. monocytogenes* to fresh-cut radish tissue and determined that flagella and the operon-encoding arabitol transport functions were necessary for that attachment process *(17)*. We sought to extend our study of the *L. monocytogenes*–plant interactions to a preharvest situation and have begun to assess the growth of the bacterium on alfalfa sprouts. In this study, several strains of *L. monocytogenes* were selected so that varying sources, serotypes, and lineages (as determined by ribotyping) were represented to see if any pattern could be discerned in their abilities to colonize alfalfa sprouts. In addition, the interaction of *L. monocytogenes* with the sprout tissue was visualized microscopically with a strain carrying a plasmid encoding the green fluorescent protein (GFP).

MATERIALS AND METHODS

Bacterial strains, plasmids, media, culture, and electroporation conditions. The *L. monocytogenes* strains, isolation sources, and serotypes are listed in Table 1. The strains were selected to represent both plant, animal, and human isolates of various serotypes. In addition, strains representing the three recognized genetic lineages, as determined by ribotyping, of *L. monocytogenes* were selected without regard for their serotype or source *(31)*. Serotypes were determined either by a reference laboratory or by the enzyme-linked immunosorbent assay–based method us-

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TABLE 1. *Strains used in this study*

Strain	Original designation	Serotype	Isolation source
L. monocytogenes			
RM2194	10403	1/2a	Clinical, human
RM2387	NA[a]	4b	Mint
RM2388	NA	1/2b	Mint
RM2707	NA	1/2b	Cheese
RM2992	2223	4b, 4d/4e[b]	Cucumber
RM2999	NA	4b	Soil
RM3000	NA	1/2c	Soil
RM3100	H7550	4b	Hot dog, epidemic
RM3153	TS29/F2365	4b	Food, epidemic
RM3155	FSL-J2-064	1/2b	Cow
RM3160	FSL-C1-056	1/2a	Human, sporadic
RM3165	FSL-J2-063	1/2a	Sheep
RM3168	LM36	4a, 4c[b]	Human, sporadic
RM3169	FSL-J1-168	4a, 4c[b]	Human, sporadic
RM3171	DD 6824	4a	Unknown
RM3183	TS21/L4486j	4b	Food, epidemic
RM3184	TS4/F6854	1/2a	Hot dog
E. coli			
DH10B		NA	NA
Plasmid			
pNF8		NA	NA

[a] Not applicable. These strains were isolated in our laboratory.
[b] Two different serotyping methods (first listed is from agglutination and the second is from enzyme-linked immunosorbent assay) give two different serotypes for these strains.

ing a commercially available antisera kit (Denka Seiken, Tokyo, Japan) *(24)*. *L. monocytogenes* was grown at 30°C in tryptic soy broth without dextrose (Difco, Becton Dickinson, Sparks, Md.) and 0.6% yeast extract. A selective medium that inhibits gram-negative and most gram-positive bacteria, modified Oxford agar (MOX; Difco) was used to assess the number of *L. monocytogenes* cells on sprouts. The motility of strains was assessed after overnight growth at 30°C in motility agar (1% tryptone, 0.5% NaCl, and 0.5% agar). *Listeria* minimal medium was made as previously described and supplemented with 40 mM arabitol as a carbon and energy source *(27)*. To obtain *L. monocytogenes* expressing GFP, pNF8 was electroporated into cells treated with penicillin and lysozyme in a modification of the method of Park and Stewart *(15, 25, 26)*. After the washing of the penicillin-treated cells described by Park and Stewart, the cells were resuspended in 10 ml of sucrose electroporation buffer (1 mM HEPES, pH 7.0, 0.5 M sucrose), and 100 μl of a 10-mg/ml lysozyme solution was added. The suspension was incubated for 20 min at 37°C and centrifuged at 3,600 × *g* for 10 min at 4°C, and the cells were washed with 10 ml of sucrose electroporation buffer. The cells were pelleted by centrifugation and then finally resuspended in 200 to 300 μl of electroporation buffer. One hundred microliters of the cell suspension and 2 μl of plasmid solution were electroporated with a Gene Pulser II (Bio-Rad, Hercules, Calif.) within 15 minutes of the preparation of the cells. The electroporation conditions were 1 kV, 400 Ω, and 25 μF in a 0.1-cm gap size cuvette. The pulse duration was approximately 5 ms. After electroporation, 1 ml of brain heart infusion medium (Difco) plus 0.5 M sucrose was added to the suspension; the culture was then incubated at 37°C for 1 h without shaking and plated onto selective medium. The plasmid pNF8 is a high-copy-number plasmid, which encodes GFP from the strong, constitutive promoter of the *L. monocytogenes dlt* operon, involved in modification of lipoteichoic acids, and has been previously described *(15)*.

To maintain pNF8, erythromycin (1 μg/ml) and lincomycin (25 μg/ml) were used for *L. monocytogenes*, and 300 μg/ml of erythromycin was used for *E. coli*. *E. coli* was grown in Luria-Bertani medium (1% tryptone, 0.5% yeast extract, 0.5% NaCl) at 37°C. For agar plates, 1.5% agar was used. Phosphate-buffered saline (PBS) contained 150 mM NaCl and 10 mM sodium phosphate (pH 7.2).

Assay for *L. monocytogenes* growth on sprouts. Experiments using sanitized seed were performed as previously described *(10)*. Alfalfa seeds (International Specialty Supply, Cookeville, Tenn.) were mixed in a ratio of approximately 1 g of seed to 5 ml of 3% $Ca(OCl)_2$ in a 50-ml conical tube and allowed to shake at room temperature for 15 min. For unsanitized seeds and sprouts, the seed was incubated in sterile water rather than $Ca(OCl)_2$ but otherwise treated identically to the sanitized seed. After washing five times with distilled water, approximately 35 seeds were placed in sterile 100 by 150-mm petri plates with 20 ml of sterile water and incubated on a bench top rotator for 4 h at approximately 40 rpm (a setting of 3 on ''The Belly Dancer,'' Stovall Life Science, Inc., Greenboro, N.C.). The water was removed and replaced with a 20-ml suspension of *L. monocytogenes* cells (10^4 CFU/ml unless indicated otherwise) in water and incubated on the shaker for another hour before replacing the *L. monocytogenes* suspension with 20 ml of sterile water. In experiments where the irrigation water was changed, sterile water was used to replace the old water daily, and the change was performed approximately 10 to 30 min before sampling of the sprouts. All sprout experiments were performed at room temperature. Sprouts were sampled by removing a sprout to 300 μl of sterile PBS and

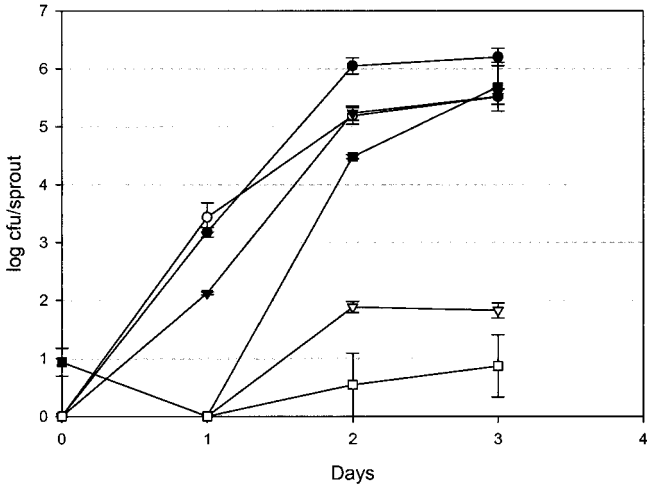

FIGURE 1. *Growth of different* L. monocytogenes *strains on alfalfa sprouts with and without regular water changes. Seeds were exposed to 10^4 CFU/ml of* L. monocytogenes *cells for 1 h on day 0. For experiments with regular water changes, the sprouts were sampled 10 to 30 min after the water change. Open symbols, experiments with regular water changes; closed symbols, no water changes; circles, RM2387; triangles, RM2194; squares, RM2992. Error bars indicate standard deviation.*

homogenizing with a sample pestle (Scienceware, no. F19922-0001, BelArt, Pequannock, N.J.) attached to a cordless rotary tool (Dremel, Racine, Wis.) operated at 7,500 rpm for approximately 2 to 3 s. In experiments where the sprouts were further washed before homogenization, 10 to 30 min after changing the irrigation water, a sprout was placed in a test tube (12 by 75 mm) containing 2 ml of sterile water and vortexed for 5 to 10 s before placing into PBS to be ground. The homogenized sprout suspensions were dilution plated onto MOX agar, and the plates incubated at 30°C for 2 days before counting of the *Listeria* colonies, which appeared bluish with a black precipitate.

Assay for growth in irrigation water. The seeds were treated with $Ca(OCl)_2$ as described above and exposed to 10^4 CFU/ml *L. monocytogenes* for 1 h. The inoculum was replaced with sterile water, and the sampling for the day 0 time point was performed within 30 minutes. On subsequent days, the water was sampled just before its replacement. Serial dilutions of the water samples were made in PBS and plated onto MOX for enumeration of *L. monocytogenes.*

Microscopy. Alfalfa seeds were sanitized and exposed to *L. monocytogenes* strain RM2387 carrying pNF8 as explained above. Erythromycin and lincomycin were present throughout the growth of sprouts inoculated with GFP containing bacteria to maintain selective pressure for pNF8. The sprouts were observed with a Leica MZ-FLIII fluorescence stereomicroscope (Leica Microsystems, Heidelberg, Germany). For observation of green fluorescent bacteria on the sprouts, a 41017 Endow GFP filter set was used (Chroma Technology Corp., Brattleboro, Vt.). Images were obtained with a Sony DKC-5000 digital camera (Sony Electronics, Inc., Tokyo, Japan) and imported into Adobe Photoshop (Adobe Systems, Inc., San Jose, Calif.) from a Sony DKS-5000 workstation.

Statistical analysis. All experiments were performed at least three times with three or four replicates each. All data shown are means of representative experiments, unless otherwise indicated, with standard deviations. Student's *t* test was used to assess confidence intervals.

TABLE 2. L. monocytogenes *present on sanitized alfalfa sprouts after 3 days of incubation at room temperature with and without daily changes of irrigation water*

Strain	No water changes[a]	Daily water changes[a]	Colonization efficiency level[b]
RM2387	6.16 ± 0.11	5.23 ± 0.31	1
RM2707	6.04 ± 0.06	5.16 ± 0.31	1
RM2999	5.43 ± 0.27	4.73 ± 0.56	2
RM3000	6.02 ± 0.19	3.86 ± 0.31	3
RM3100	5.88 ± 0.21	3.74 ± 0.59	3
RM2194	5.72 ± 0.35	2.53 ± 1.16	4
RM2388	5.45 ± 0.37	2.95 ± 1.10	4
RM2992	5.72 ± 0.31	0.42 ± 0.51	5

[a] Values are number (log CFU per sprout) ± standard deviation.
[b] Level of efficiency determined by statistical analysis.

RESULTS

***L. monocytogenes* growth on alfalfa sprouts.** Alfalfa seeds were treated with $Ca(OCl)_2$, rinsed, and exposed to 10^4 CFU/ml of different strains of *L. monocytogenes* in 20 ml of water for 1 h. In no case did the presence of *L. monocytogenes* impair the growth of the sprouts. A time course of representative experiments for the growth for three strains on sprouts with and without daily water changes is shown in Figure 1. At day 0, sampled shortly after the replacement of the inoculation suspension, there was less than 1 log CFU of *L. monocytogenes* detectable on all of the seeds. Without water replacement, most strains showed some growth on the sprouting seeds by day 1 (approximately 2 to 3 log CFU per sprout) and reached a peak of 5 to 6 log CFU per sprout at day 2. The levels of *L. monocytogenes* at day 3, when the sprouts were mature, were similar to those at day 2. Figure 1 also illustrates examples of the three strains grown on sprouts when the water was replaced daily. At its peak, there was a 1-log reduction in the amount of RM2387 on sprouts when the water was changed regularly. This result is contrasted by strain RM2194, which was reduced by 3 log from the RM2387 levels on sprouts as a result of the water changes. The effect of the water change was further enhanced in RM2992, which grew to less than 1 log on sprouts subjected to water changes, despite reaching levels of more than 5 log on sprouts with no water changes.

Table 2 displays the results of the growth of different strains on sprouts grown for 3 days under both conditions. The numbers in Table 2 are averages from multiple experiments. When there was no change of irrigation water, all strains of *L. monocytogenes* grew to between 5 and 6 log CFU per sprout after 3 days of growth. The levels of *L. monocytogenes* on the sprouts were monitored daily, and the growth kinetics of all the strains on sprouts grown without regular water changes were similar to those shown in Figure 1 (closed symbols). Differences among the strains were evident when the irrigation water was changed. When the water was replaced daily, the levels of association of

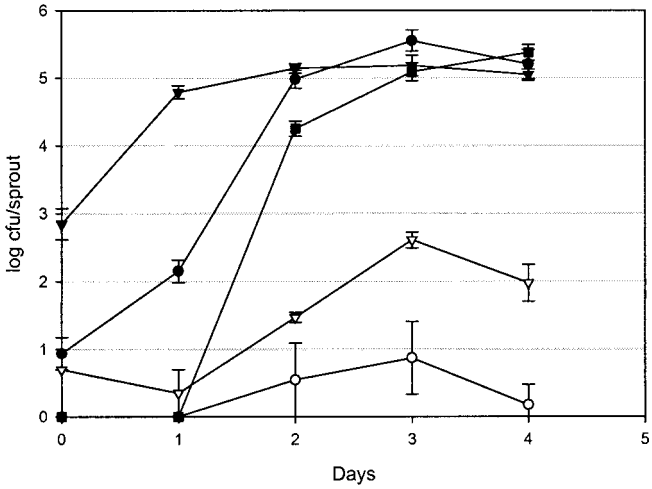

FIGURE 2. *Numbers of cells of strains RM2387 and RM2992 on alfalfa sprouts inoculated with varying cell densities. Sanitized seeds were exposed for 1 h to* L. monocytogenes *suspensions at the indicated densities. The numbers of* L. monocytogenes *on the sprouts grown with daily water changes was measured. Closed symbols, RM2387; open symbols, RM2992. Inoculating cell densities: squares, 10^2/ml; circles, 10^4/ml; triangles, 10^6/ml. Error bars indicate standard deviation.*

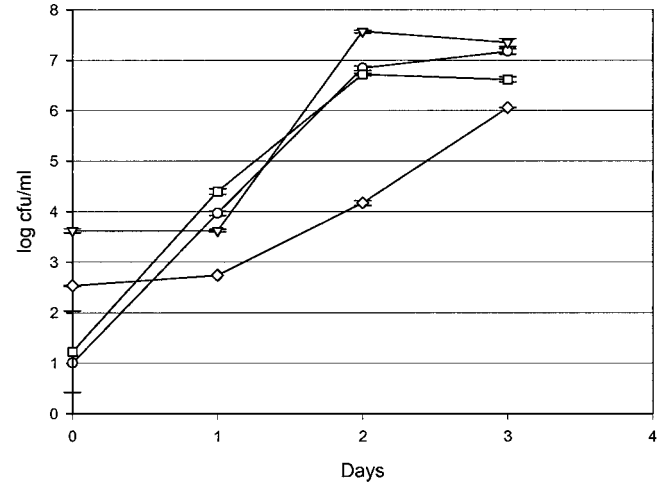

FIGURE 3. *Growth of* L. monocytogenes *strains in the irrigation water of alfalfa sprouts. The irrigation water was changed daily. The water was sampled just before changing. Circles, RM2194; triangles, RM2387; squares, RM2707; diamonds, RM2992. Error bars indicate standard deviation.*

some strains of *L. monocytogenes* were close to zero, whereas others were within a log of the numbers attained without water changes. Statistical analysis indicated that the strains could be divided into five levels based on their efficiency of colonization of the sprouts with regular water changes ($P < 0.05$). Strains RM2387 and RM2707 comprised level 1 in colonization efficiency, reaching between 5 and 6 log CFU per sprout by day 3. Level 2 had one member, RM2999, which reached between 4 and 5 log CFU per sprout. Level 3, which was composed of RM3000 and RM3100, reached between 3 and 4 log CFU per sprout, respectively. Strains RM2194 and RM2388 fell into level 4, with levels between 2 and 3 log CFU per sprout, respectively, and finally, RM2992 was in level 5, reaching less than 1 log CFU per sprout after 3 days of growth.

Effect of inoculum size on levels of growth. Two strains were analyzed further to determine if the number of cells in the initial inoculum would affect the final number of *L. monocytogenes* reached on the sprouts (Fig. 2). Specifically, we wondered if the poor level 5 colonizer RM2992 would colonize better if more cells were used for inoculum and if the good level 1 colonizer RM2387 would reach even higher levels under the same conditions. For these experiments, the irrigation water was changed daily. The level of growth for the level 1 colonizer RM2387 after 4 days was independent of the amount of cells used to inoculate the seeds on day 0 (1-h exposure of 10^2, 10^4, or 10^6 CFU/ml). The final amounts that resulted from all the inocula reached over 5 log CFU per sprout. Increasing the inoculum size from 10^4 to 10^6 CFU/ml on day 0 affected the number of level 5 colonizer RM2992 detected on the sprouts, with roughly 1.5 log higher CFU per sprout at day 3. However, this peak was reduced approximately 3 log from that of RM2387. Also, there was no increase in the number of cells of RM2992 on the sprouts when the experiment was increased in length to 4 days. In fact, the numbers of cells decreased slightly for each inoculation level.

Growth of strains in the irrigation water. The number of RM2992 cells in the irrigation water was examined to determine whether this was related to the low numbers of that strain seen on the resulting sprouts. The results for RM2992 were compared with three other strains to compare the poor level 5 colonizer with both good colonizing level 1 strains (RM2387 and RM2707) and its nearest neighbor in the colonization ladder, the level 4 strain RM2194 (Fig. 3). There was variance among the strains on day 0 regarding how many cells were present in the water; however, there was less variability by day 1. In all cases, the amounts of *L. monocytogenes* reached at least 6 log CFU/ml by day 3 of the experiment. RM2387 had the highest initial amount at day 0 (3.6 log CFU/ml) and stayed level through day 1 before reaching a peak of 7.5 log CFU/ml at day 2. RM2707 started at approximately 1 log CFU/ml and reached its peak of 6.7 log CFU/ml at day 2. RM2194 also started at approximately 1 log CFU/ml and grew to a peak of more than 7 log CFU/ml at day 3. In these three cases, the growth leveled off after day 2. The rate of growth of RM2992 was slower than for the other three strains; nevertheless, it reached 6 log CFU/ml by day 3.

***L. monocytogenes* growth on unsanitized sprouts and the effect of additional washing.** Level 1 colonizers RM2387 and RM2707 and level 4 colonizer RM2194 were tested to determine whether they became associated with sprouts that were not pretreated with $Ca(OCl)_2$. These strains were selected to see if there were differences among good and bad colonizers with respect to the presence of normal sprout flora. The data presented in Table 3 represent a compilation from multiple experiments. After 3 days of incubation with daily changes of irrigation water, there were

TABLE 3. *Attachment of three strains of* L. monocytogenes *to sanitized and unsanitized alfalfa sprouts and the effect of additional washing*[a]

Strain	Sanitized sprouts	Unsanitized sprouts	Sanitized and additional wash
RM2387	5.24 ± 0.21	5.17 ± 0.26	4.87 ± 0.12
RM2707	5.39 ± 0.23	5.03 ± 0.52	4.28 ± 0.39
RM2194	2.41 ± 0.21	2.49 ± 0.14	<0.1[b]

[a] Values are log CFU per sprout ± standard deviation.
[b] Lower limit of the assay.

no significant differences within a given strain between the amount of cells present on either the sanitized or unsanitized sprouts ($P > 0.05$).

The three strains were tested further to see if they could be rinsed off the sprouts by adding a washing step after the change of the irrigation water. The results from multiple experiments are shown in Table 3. Additional washing decreased the number of RM2387 and RM2707 on sanitized alfalfa sprouts by 0.3 and 1.1 log CFU per sprout, respectively. These reductions were statistically significant ($P < 0.05$). There were no detectable cells of RM2194 on sprouts that were washed. The lower limit of the assay was less than 0.1 log CFU per sprout.

Assessment of lineage as a factor in sprout attachment. Strains were chosen to assess if differences in sprout attachment could be attributed to genetic lineage. The results are shown in Table 4. No statistical trends could be seen either within or between lineages regarding the numbers attached to sanitized sprouts after 3 days of growth with daily water changes. With one exception, all of the strains attached at levels between 3.37 and 5.22 log CFU per sprout. Strain RM3153 grew to 2.67 log CFU per sprout. Statistical analysis revealed that within the lineage II and III strains, all the attachment values were equivalent ($P > 0.05$). Within lineage I strains, RM3153 was statistically different from both the other lineage I strains, RM3155 and RM3183, and the lineage II and III strains tested here ($P < 0.05$).

TABLE 4. *Attachment of* L. monocytogenes *strains of different lineages to sanitized alfalfa sprouts after 3 days*

Strain	Lineage	Number attached[a]
RM3153	I	2.67 ± 0.54
RM3155	I	4.78 ± 0.59
RM3183	I	5.22 ± 0.27
RM3160	II	4.18 ± 0.12
RM3165	II	3.37 ± 0.76
RM3184	II	3.97 ± 0.47
RM3168	III	4.21 ± 0.25
RM3169	III	4.38 ± 0.05
RM3171	III	4.96 ± 0.26

[a] Values are log CFU per sprout ± standard deviation.

Motility and growth characteristics. In a previous study, *L. monocytogenes* genes important for attachment to radish tissue were mapped to a flagellar biosynthetic operon and an arabitol transport operon. Among the mutants defective in radish attachment were those defective in motility and also growth on arabitol as a carbon source *(17)*. All of the strains used in this present study were determined to be motile by testing in motility agar. Additionally, RM2992 and RM2194 grew to the same level as RM2387 in arabitol minimal medium, indicating that transport of arabitol was functional in the strains.

Microscopy. RM2387 containing the GFP-encoding plasmid pNF8 was used to visualize *L. monocytogenes* on the alfalfa sprouts in situ (Fig. 4). Antibiotics were included in the system when microscopy was performed, because during the 3-day experiment more than 80% of the cells lost the plasmid without selective pressure (data not shown). The presence of pNF8 had no effect on the number of RM2387 present on the sprouts (data not shown). Examination of the sprouts at 2 to 3 days revealed that *L. monocytogenes* strain RM2387 was targeted to the root and seed coats of the alfalfa sprouts. No *L. monocytogenes* cells were detected on the leaves. Higher magnification showed that cells were concentrated on the root hairs of the sprout and on the edges of the seed coats as the sprout emerged.

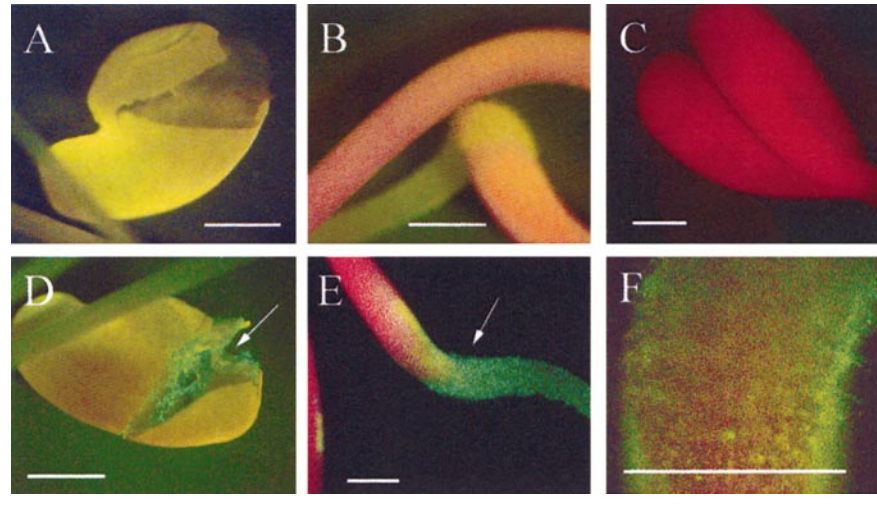

FIGURE 4. *Fluorescent stereoscope micrographs of* L. monocytogenes *strain RM2387 attached to sprouting alfalfa after 3 days of growth. (A) Uninoculated seed coat. (B) Uninoculated alfalfa root. (C) Leaf from inoculated sprouts with no detectable green fluorescence. (D) Colonized seed coat edge. (E) Colonized root. (F) High-magnification view of colonized root with* L. monocytogenes *coating the hairs. The fuzzy appearance at high magnification is due to colonization of the root hairs within different focal planes. All scale bars equal 1 mm. Arrows indicate regions of densely attached cells.*

DISCUSSION

Foodborne illness as a result of eating uncooked sprouts commonly occurs with *Salmonella* and occasionally with *E. coli (23)*. *L. monocytogenes* has been the cause of sprout recalls. Whereas no cases of listeriosis have been linked to sprouts, there have been cases linked to alfalfa tablets *(13, 23)*. Our use of sprouts to assess the growth of *L. monocytogenes* served as a model of preharvest contamination with a foodborne pathogen that is also a soil-borne organism and saprophyte. Sprouts grow quickly, are easy to manipulate in the laboratory, and allow for simple assessments of the *L. monocytogenes*–plant interaction to understand *L. monocytogenes* biology in that environment. We generally used $Ca(OCl)_2$-treated seed, because it has been recommended for use among sprout producers *(23)*.

In the present study, we assessed the growth of strains of *L. monocytogenes* of varying source, serotype, and lineage on alfalfa sprouts. Thirteen different serotypes of *L. monocytogenes* have been described, although 95% of human listeriosis cases belong to serotypes 1/2a, 1/2b, and 4b *(20)*. Further differentiation of *L. monocytogenes* has been described with the recognition of three distinct lineages based on ribotyping and virulence gene polymorphisms *(8, 19, 31)*. All the strains tested in this study were able to grow on the sprouts and the seed exudates over 3 days of growth, reaching levels of 5 to 6 log CFU per sprout. This result was similar to the amount of growth reported for various strains of *S. enterica* and *E. coli* O157:H7 on 3-day-old alfalfa sprouts *(10)*. Fett *(14)* reported that laboratory-grown alfalfa sprouts contained roughly 8 log CFU of mesophilic aerobic bacteria per g of sprout material. Matos et al. *(22)* reported that the average microbial cell density on alfalfa sprouts obtained from several commercial facilities was between 6.5 and 7.2 log CFU/g of sprouts. Considering that an alfalfa sprout weighs approximately 0.1 g, then the normal number of aerobic, mesophilic bacteria present on a 2- to 3-day-old alfalfa sprout would be approximately 6 to 7 log CFU. Therefore, the number of *L. monocytogenes* capable of colonizing alfalfa sprouts was approximately 1 log less than the total number of normal sprout bacteria. When the amount of inoculum was varied for RM2387, a strain that grew well on sprouts grown with and without water changes, there was no difference in the ultimate number of *L. monocytogenes* present on those sprouts (Fig. 2), suggesting that there might be a listerial load that the alfalfa sprouts can carry.

Previous reports indicated differences in the ability of *S. enterica* and *E. coli* O157:H7 to attach to alfalfa sprouts. This difference manifested itself when the irrigation water in which the sprouts were growing was changed regularly *(10)*. Sampling of unwashed sprouts measures the numbers of bacteria in the water, as well as those only peripherally associated with the plant tissue. Therefore, we decided to measure *L. monocytogenes* growth on alfalfa sprouts when the irrigation water was changed daily. The level of *L. monocytogenes* detected after 3 days under those conditions ranged widely, depending on the strain. Five discrete levels of colonization by *L. monocytogenes* could be differentiated (Table 2). The varying levels of growth on sprouts grown with regular changes in irrigation water could reflect different capabilities of the strains to attach to the sprouts.

Since the changing of the irrigation water could act as a type of wash of the sprouts, we wondered if the wide range of growth of *L. monocytogenes* strains under those conditions reflected differences in attachment of the bacteria to the plant material. Strain RM2992 was present at low levels on sprouts with daily water changes but at high levels on sprouts grown without water changes. The levels on the sprouts without regular water changes would not reflect colonization of the sprout per se but rather the number in the water and those in weak or strong association with the sprouts. All the strains tested grew similarly in the sprout water and, hence, were present with the sprouts during growth (Fig. 3). Therefore, RM2992 was present in the irrigation water at levels sufficient to visualize it on the sprouts if, indeed, it were present on those sprouts. Bacteria could persist in the experiment associated with the sprout, with the seed coats, and in the water. Similar levels of the different strains of *L. monocytogenes* strains present in the water contrasting with major differences measured on the sprouts suggest that the differences were due to variation in attachment to the sprouts. Seemingly, the interaction of RM2992 with the sprouts was so weak that the mere replacement of the growth water washed those cells off the sprouts. When the initial inoculum was increased by 100-fold, there was an increase in the level of RM2992 detected on the sprouts, but the levels were still far below those of the efficient level 1 colonizer RM2387. Increasing the experiment to 4 days had no effect on the level of RM2992 detected (Fig. 2).

In a previous study, it was determined that eight strains of *L. monocytogenes* attached to cut radishes despite multiple washings, indicating that the radish attachment trait was intrinsic for those strains. Three genes associated with radish attachment were identified in the study. Two of the identified regions were associated with phenotypes that were easily tested: motility and growth on arabitol *(17)*. The poor-alfalfa-colonizing RM2992 and the weak-colonizing RM2194 had no motility defects and were capable of growth in arabitol minimal medium, indicating that flagella were present and arabitol transport was functional in those strains. Therefore, any attachment defect for those strains in this alfalfa sprout system was not related to the genes identified in the radish study *(17)*.

Whereas the interaction of RM2992 with the sprouts was weak, strains RM2387 and RM2707 were not washed easily off the sprout tissue (Table 3). Additional washing of the weak-colonizing RM2194 led to no detectable *L. monocytogenes* cells on the sprouts. This result may indicate that the weak-colonizing strains have only a peripheral association with the sprout tissue and hence are removed easily, whereas the strong colonizers have a more formidable relationship and are not easily removed. Differences in the fitness of strain colonization are being studied currently with both microscopic and genetic approaches.

Sanitized seeds were routinely used in these experiments, so we reasoned that it was possible that a decrease

in the natural sprout microflora enhanced the ability of *L. monocytogenes* to colonize the sprout tissue. To determine whether this was occurring, we tested the two best colonizing strains, RM2387 and RM2707, and a weak colonizer, RM2194, on unsanitized sprouts (Table 3). The data indicated that the presence of normal sprout bacteria did not affect the ability of *L. monocytogenes* to colonize the sprouts. Even though *L. monocytogenes* can live in the soil and associates with plants, this result was surprising. It suggests that, given the opportunity, *L. monocytogenes* can compete well with normal resident plant microflora. It should be noted that Barak et al. *(7)* reported that *S. enterica* strains competed well with normal sprout bacteria for adherence to mature alfalfa sprouts, with *S. enterica* adhering in equivalent numbers to *Pantoea agglomerans* and 10 times better than *Pseudomonas putida* and *Rahnella aquatilis*.

Microscopy with *L. monocytogenes* containing a plasmid encoding a constitutively active GFP indicated that the bacteria targeted the roots of the plant for colonization, despite the fact that the entire sprout was immersed in water, shaking throughout the experiment (Fig. 4). This result indicated that the root hairs provided the preferential niche for *L. monocytogenes* on sprouting alfalfa. It has been reported previously that both *S. enterica* and *E. coli* O157:H7 attach to similar areas on growing alfalfa sprouts, indicating that these human pathogens can interact with plant tissue *(10)*.

Serotype is a function of different surface characteristics of a bacterium, leading to variable interactions with different antisera to whole cells *(21, 30)*. Therefore, it was reasonable to test whether different serotypes of *L. monocytogenes*, reflecting different cell surface chemistry, would play a role in attachment or colonization. Similarly, the source of an isolate might be important, with plant isolates better suited for plant colonization. RM2992, the poorest colonizer, was isolated from a plant sample and belonged to serotype 4b or 4d/4e (depending on the serotyping method used) *(24)*. RM2387, a level 1 colonizer, was also a plant isolate and serotype 4b. However, RM2707, another level 1 colonizer, is a serotype 1/2b strain originally isolated from cheese. The remainder of the strains represented serotypes 4b, 1/2a, 1/2b, and 1/2c and were isolated originally from soil, meat, plants, animals, or humans. According to the strains selected for this study, source and serotype were found not to play a role in the colonization. The role of strain lineage (which is not necessarily a reflection of cell surface variation) was assessed directly with the testing of nine strains of known lineage (Table 4) *(31)*. No discernible patterns in strain attachment could be related to serotype, source, or lineage. One lineage I strain, RM3153, attached at lower levels than the others of known lineage shown in Table 4. We believe the lower level of attachment for this strain reflected what appeared to be the strain variance seen with the other strains tested in the study and not differences related to lineage, since the other two lineage I strains were not significantly different from the lineage II and III strains displayed in Table 4. All together, the data illustrated that the factors necessary for this plant interaction were neither those that determined serotype or lineage nor related to the original source of the isolate. Intuitively, a plant isolate would be thought to be best suited to growth on plants; however, the simple isolation of *L. monocytogenes* from plant tissue does not necessarily correlate with its suitability for that environment. It is possible that such an isolate was not associated directly with the plant tissue but rather present in soil remnants or associated indirectly, such as in a moisture layer or in a microcolony with other bacteria.

With regard to sprout production, our data suggest that the colonization ability of *L. monocytogenes* for alfalfa sprouts is dependent on the strain. If the sprouts are contaminated with a good attaching strain, it may prove difficult to remove attached cells through washing. Currently, we are focusing our work on the identification of the *L. monocytogenes* factors that are necessary for the alfalfa attachment and colonization process.

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